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Use of monoclonal anti-light subunit antibodies to study the structure and function of the *Entamoeba histolytica* Gal/GalNAc adherence lectin

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Adherence of *Entamoeba histolytica* trophozoites to host cells is mediated by a galactose (Gal) and *N*-acetylgalactosamine (GalNAc)-specific surface lectin. The lectin is a heterodimeric protein composed of heavy (170 kDa) and light (35–31 kDa) subunits linked by disulfide bonds. Polyclonal and monoclonal antibodies (mAb) raised against a light subunit-glutathione-*S*-transferase fusion protein were used to probe its structure and function. Four light subunit-specific mAb were produced which recognized distinct epitopes on five different light subunit isoforms. Immunoblots with these mAb demonstrated co-migration of light and heavy subunits when nonreduced trophozoite proteins were analysed by SDS-PAGE, indicating that the subunits do not exist free of the heterodimer in significant quantities. While anti-heavy subunit antibodies had previously been shown to alter adherence, anti-light subunit antibodies did not, suggesting that the heavy subunit contains the carbohydrate recognition domain.

Keywords: lectin; *Entamoeba histolytica*; *Entamoeba dispar*; adherence; galactose; amebiasis.

Introduction

Glycoconjugate-lectin interactions play an important role in the pathogenesis of amebiasis. Amebiasis is a common worldwide parasitic infection that results annually in 40 to 50 million cases of amebic colitis and liver abscess. Trophozoites adhere to human colonic mucins and colonic epithelial cells to initiate colonization and invasion of humans. Adherence *in vitro* is mediated by a surface lectin that binds to terminal galactose (Gal) and *N*-acetylgalactosamine (GalNAc) [1, 2]. *Entamoeba histolytica* kills human macrophages, monocytes, neutrophils and T lymphocytes in a contact-dependent process that also requires the activity of this lectin. Mere apposition of amebic and target cell plasma membranes, as can be achieved by centrifuging target cells and amebae together into a pellet, will not lead to cytolysis if the amebic lectin is inhibited with Gal or GalNAc, indicating that the lectin either signals the initiation of cytolysis or directly participates in the cytolytic event.

Evasion of serum lysis also involves the Gal/GalNAc lectin: evasion of the host complement system is critical for survival of the extracellular trophozoite. *Entamoeba histolytica* trophozoites are resistant to the complement C5b-9 complexes which form the membrane attack complex. Monoclonal antibodies directed against amino acids 895–1082 of the cysteine-rich domain (epitopes 6 and 7) of the lectin heavy subunit greatly increase the sensitivity of *E. histolytica* to lysis by human sera and by purified human C5b-9. The lectin binds to purified human C8 and C9, and the binding is inhibited by anti-lectin mAb which block serum resistance. The purified lectin confers C5b-9 resistance when reconstituted into C5b-9 sensitive amebae, a direct demonstration of its C5b-9 inhibitory activity [3, 4].

The amebic Gal/GalNAc lectin is a 260 kDa heterodimeric glycoprotein consisting of heavy (170 kDa) and light (35 and 31 kDa) subunits linked by disulfide bonds [5]. The 170 kDa subunit is encoded by a gene family of which three members (89–95% identical) from *E. histolytica* strain HM1:IMSS have been sequenced [6]. The 170 kDa subunit sequences contain a carboxy-terminal putative cytoplasmic and transmembrane domain followed by an extensive extracellular cysteine-rich domain. This cysteine-rich domain is recognized by adherence-inhibitory anti-lectin monoclonal antibodies (mAb) [4].

The light subunit has been resolved into 35 and 31 kDa isoforms by SDS-PAGE. The 35 and 31 kDa isoforms have

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nearly identical amino acid compositions and CNBr-fragmented peptide patterns. Two light subunit genes have been sequenced from strain HM1:IMSS, both of which encode proteins (80.6% identical) with potential GPI anchor addition sequences [7, 8]. The 31 kDa isoform contains a GPI anchor, as demonstrated by the release of phosphatidylinositol with nitrous acid [5]. The heterodimeric structure of the lectin is apparently unique, with one subunit transmembrane and one GPI-anchored.

The locations of the carbohydrate and complement recognition sites of the lectin are unknown. The conserved carbohydrate-binding motifs present in the eukaryotic C and S type lectins, the *E. coli* gal-gal binding lectin and plant lectins are not found in the sequence of either subunit. Direct assignment of galactose-binding activity to either the 170 kDa or 35 kDa subunits of the native lectin has not been possible, as the reduction in disulfide bonds required to separate the subunits results in loss of galactose-binding activity (Petri, unpublished results). However, the ability of mAb directed against the cysteine-rich domain of the 170 kDa subunit (amino acids nos 596–1082) to enhance and inhibit the galactose-binding activity of the protein suggests that the 170 kDa subunit contains the carbohydrate binding region [4].

Here we report the production of monoclonal antibodies (mAb) against the light subunit of the Gal/GalNAc lectin and their use in its structural and functional characterization.

Materials and methods

Cultivation and harvesting of E. histolytica *trophozoites and CHO cells*

Axenic *E. histolytica*, strain HM-1:IMSS and nonaxenized *E. dispar*, strain SAW 760 (a kind gift from Dr David Mirelman, Weizmann Institute, Rehovot, Israel), were grown in medium TYI-S-33 (trypticase and yeast extract, iron and serum) with 100 units per ml penicillin and 100 μg ml^{-1} streptomycin sulphate at 37 °C in 15 ml glass culture tubes [9]. Amebic trophozoites were harvested after 72 h of growth by centrifugation at 150 × **g** for 5 min at 4 °C and washed in 75 mM Tris (Sigma, St Louis, MO), 65 mM NaCl, pH 7.2 [1]. Chinese hamster ovary (CHO) cells were grown in F-12 medium (GIBCO) with 10% fetal bovine serum with 100 units per ml penicillin and 100 μg ml^{-1} streptomycin sulphate at 37 °C in a water-jacketed CO_2 incubator and harvested by trypsinization.

SDS-PAGE

SDS-PAGE was performed by the method of Laemmli [10]. Molecular weight determinations were made using Rainbow Markers (14 300–200 000) from Amersham. Trophozoites (10^5 per lane) in 75 mM Tris, 65 mM NaCl, pH 7.2, were solubilized by adding one volume of boiling 2X SDS-PAGE sample buffer (containing 4% SDS, 10% β-mercaptoethanol, 5 mM EDTA, 2 mM *p*-hydroxymercuribenzoate, and 2 mM phenylmethylsulfonylfluoride [Sigma, St Louis, MO]) to one volume of amebic trophozoites.

Immunoblotting

Five μg per lane of affinity-purified lectin or 10^5 trophozoites per lane from reducing SDS-PAGE were electrophoretically transferred to PVDF membranes for Western blots. The excess protein-binding capacity of the membranes was blocked with 5% nonfat dry milk (Carnation, Los Angeles, CA) in blot wash buffer (50 mM Tris, 200 mM NaCl, pH 7.5, 0.1% Tween 20) for at least 1 h. The membranes were then incubated for 1 h at room temperature with mouse antiserum (diluted 1:1000), mAb (10 μg ml^{-1}), or mAb culture supernatant (1:10), washed 3 × 10 min, with the first wash at 50 °C, and incubated for 1 h with an anti-mouse IgG alkaline phosphatase conjugate diluted 1:7500 (Promega, Madison, WI). Immunoblots were washed 3 × 10 min and developed in Western Blue substrate (Promega).

ELISA

Wells of 96-well microtiter plates (flat-bottomed polystyrene ELISA plates: Costar, Cambridge, MA) were coated with purified galactose-specific lectin by overnight incubation of 0.1 μg of lectin in 100 μl of coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$ 0.003 M NaN_3 at pH 9.6) at 4 °C. Wells were washed twice with PBS-Tween-1% BSA and incubated with 1% BSA in coating buffer for 2 h at room temperature to prevent nonspecific binding to polystyrene. Test sera were added at desired dilutions in PBS-Tween-1% BSA and incubated overnight at 4 °C. Wells were washed four times with PBS-Tween, and incubated for 2 h at room temperature with 100 μl of anti-mouse IgG alkaline phosphatase conjugate (1:7500 in PBS-Tween-1% BSA). Wells were washed five times with PBS-Tween before 100 μl per well of enzyme substrate (1X diethanolamine buffer, p-nitrophenyl phosphate tablets, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added. Optical density was measured in an ELISA plate reader at 405 nm.

Immunization of mice with light subunit constructs

The production of the GST-LGL1 fusion protein has previously been reported [9]. A light subunit peptide was synthesized base on the derived amino acid sequence of *lgl1*. A cysteine residue was linked to the amino terminus of the peptide to allow conjugation to Keyhole Limpet hemocyanin. The peptide (CTDEDDKKGDE) (amino acids 83–92) was synthesized at the Protein and Nucleic Acid Sequencing Facility at the University of Virginia and conjugated to maleimide activated Keyhole Limpet hemocyanin using the Pierce Imject™ activated immunogen conjugation kit according to the manufacturer's instructions. The conjugate was purified by gel filtration. Balb/C (Hilltop Laboratories) male (6–8 weeks) mice were initially

immunized with 50 μg of peptide-KLH conjugate or GST-LGL1 fusion protein, 150 μl of complete Freund's adjuvant, and phosphate-buffered saline (PBS), pH 7.4, for a final volume of 300 μl per animal. At 2 week intervals, the animals were boosted with 50 μg of antigen, 150 μl of incomplete Freund's adjuvant, and PBS (final volume of 300 μl per animal). All immunizations were performed intraperitoneally (ip). Mice were bled prior to each immunization. Serum antibody production was analyzed by ELISA and Western blotting.

Development of light subunit-specific monoclonal antibodies

Balb/C mice immunized with the GST-LGL1 fusion protein received a final intrasplenic boost with 50 μg of antigen before the spleen cells were isolated and fused to $S_p2/0$-Ag14 myeloma cells with polyethylene glycol and antibody-producing hybrids isolated as previously described [11]. Anti-lectin antibody producing hybridomas were identified using an enzyme-linked immunosorbent assay with amebic lectin-coated microtiter plates [12].

Adherence assays

Adherence to CHO cells was measured by the method of Ravdin and Guerrant [1]. Anti-lectin mAb (protein A or G purified from ascites) were preincubated with trophozoites at a concentration of 10 μg per 10^4 amebae for 45 min at 4 °C. Amebae (10^4) and CHO cells (2×10^5) were pelleted together at 4 °C; for adherence assays they were incubated for 60 min at 4 °C prior to resuspension of the pellet and assessment of adherence by rosette formation [1].

Results and discussion

The Gal/GalNAc lectin purified by affinity chromatography with lectin heavy subunit-specific mAb consists of major heavy subunit bands (at approximately 170 kDa) and light subunit bands of 35 and 31 kDa (Fig. 1). Previously we showed that the 35 and 31 kDa lectin light subunit bands have similar amino acid compositions and CNBr peptide patterns but differ in post-translational modifications [5].

Four anti-light subunit antibody producing hybridomas (designated 1C8, 1A9, 1D4 and 3C2; IgG isotopes 2b, 1, 2a and 2a respectively) were successfully subcloned from a Balb/C mouse immunized with the bacterially-expressed glutathione-*S*-transferase-35 kDa light subunit isoform (GST-LGL1) fusion protein. These hybridomas were identified by screening for the production of antibodies which recognized the affinity-purified lectin coated on microtiter wells.

The mAb and polyclonal antibodies produced against the GST-LGL1 light subunit fusion protein, as well as anti-peptide antisera produced to a peptide encompassing LGL1 amino acids 83–92, were used in Western blots to analyze their reactivity with the light subunit isoforms of affinity-purified Gal/GalNAc lectin (Fig. 2). As previously reported, anti-GST-LGL1 antiserum reacted with both 35 and 31 kDa light subunit isoforms [9]. In addition, at least three distinct bands were seen in the 35 kDa region (Fig. 2, lane

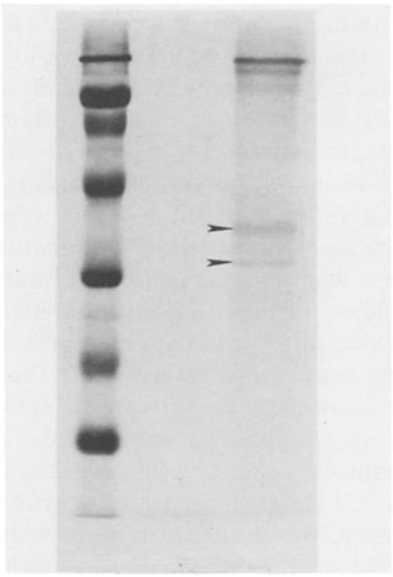

Figure 1. SDS-PAGE of monoclonal antibody affinity purified lectin. The purified lectin (right lane) was electrophoresed in a 12% polyacrylamide gel in the presence of 2-mercaptoethanol. Positions of the 35 and 31 kDa light subunit isoforms are marked with arrowheads. Staining was performed with Coomassie Blue. Molecular mass markers are 200, 92, 69, 46, 30, 21 and 14 kDa (left lane).

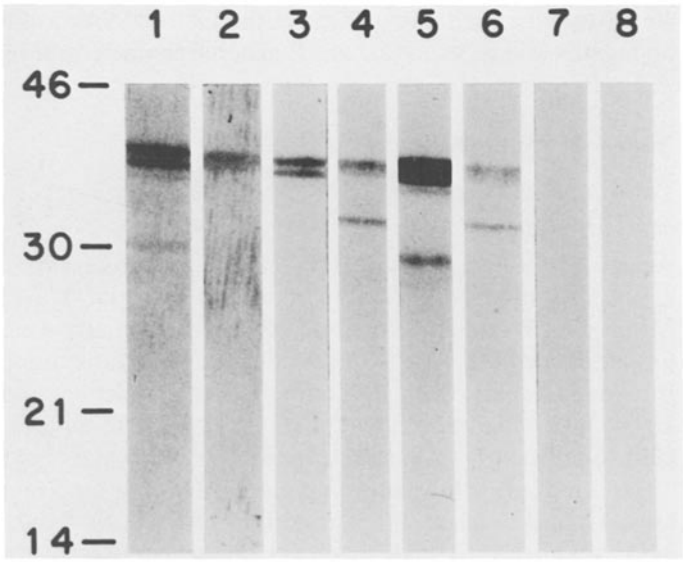

Figure 2. Western blots of affinity-purified lectin with anti-light subunit antisera and mAb. Affinity-purified lectin was electrophoresed by SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were immunoblotted with: (1) mouse anti-GST-LGL1 antiserum; (2) mouse anti-peptide (LGL1 amino acids 83–92) antiserum; or the following anti-GST-LGL1 mAb: (3) 1A9: (4) 1C8; (5) 1D4; (6) 3C2; (7) pre-immune serum (1:1000); (8) secondary antibody only.

1). The antipetide antisera also recognized multiple light subunit bands (Fig. 2, lane 2). These bands may represent different post-translational modifications or proteolytic processing of the 35 kDa isoform, or distinct proteins encoded by different members of the light subunit gene family, which is composed of a minimum of four different genes (G. Ramakrishnan and W. Petri, unpublished).

Anti-light subunit mAb 1A9, 1C8, and 1D4 differed in their patterns of recognition of the 35 and 31 kDa isoforms on immunoblots of reduced lectin. Monoclonal antibody 3C2 had an identical pattern of recognition as mAb 1C8, binding to 35 and 33 kDa bands but not to a 31 kDa light subunit band (Fig. 2, lanes 3–6). These three different patterns of reaction with light subunit bands by the 4 mAb indicated that at least three distinct light subunit protein epitopes were recognized, with the three epitopes not conserved on all of the light subunit bands resolved by SDS-PAGE. The complexity of the Gal/GalNAc lectin light subunit is revealed by these anti-light subunit mAb, with five antigenically distinct bands of 35–31 kDa identified. No reactivity was seen with mouse pre-immune serum or with the secondary antibody alone (Fig. 2, lanes 7 and 8). None of the anti-light subunit antibodies cross-reacted with the 170 kDa lectin subunit (data not shown).

To determine if the light subunit is always found in association with the lectin heavy subunit in trophozoites. Western blots of total amebic proteins were performed using the light and heavy subunit-specific mAb. In the absence of 2-mercaptoethanol the heterodimeric lectin migrates in SDS-PAGE as a single band with an approximate mass of 260 kDa [11]. Western blots of nonreduced total *E. histolytica* proteins with anti-heavy and anti-light subunit mAb revealed a single band of approximately 260 kDa (Fig. 3), lanes 2–3). The demonstration that the heavy and light subunits co-migrate in the nonreduced state is consistent with the accepted subunit structure of the lectin [12] and does not support the light subunit participating in other heterodimers or existing as a monomer in the trophozoite.

Entamoeba dispar is a closely related nonpathogenic ameba which contains a Gal/GalNAc lectin. The *E. dispar* lectin contains a 170 kDa heavy subunit which cross-reacts with *E. histolytica* anti-heavy subunit mAb [13, 14]. To test if *E. dispar* contained proteins that cross-reacted with the light subunit-specific mAb, Western blots of total *E. dispar* proteins reduced with 2-mercaptoethanol were performed (Fig. 3, lanes 6–7). The mAb against the *E. histolytica* light subunit cross-reacted with *E. dispar* protein bands of estimated mass of 34 and 32 kDa (Fig. 3, lanes 6–7) which did not co-migrate with the light subunit bands of *E. histolytica*. Under nonreducing conditions light and heavy subunit-specific mAb both recognized an *E. dispar* band of approximately 260 kDa (data not shown), consistent with similar subunit compositions and structure of the *E. dispar* lectin.

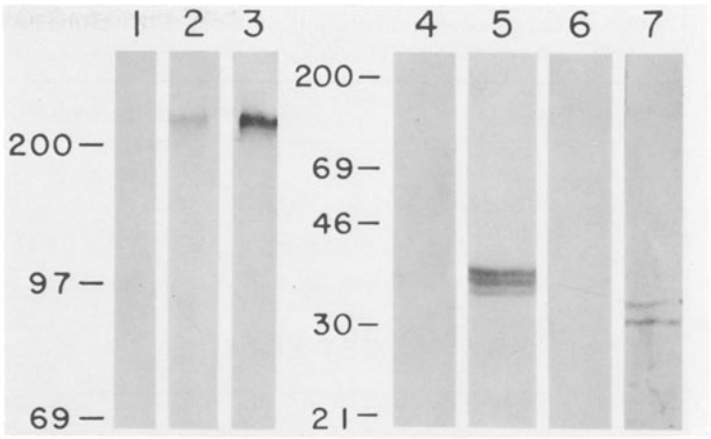

Figure 3. Western blots of amebic trophozoites with anti-lectin antibodies. Lanes 1–3: *E. histolytica* trophozoites (10^5 per lane) were electrophoresed by SDS-PAGE in the absence of 2-mercaptoethanol, transferred to PVDF membranes and probed with: (1) secondary antibody alone; (2) anti-heavy subunit mAb; or (3) a cocktail of all 4 anti-light subunit mAb from Fig. 2. Lanes 4–5: *E. histolytica* trophozoites (10^5 per lane) electrophoresed by SDS-PAGE with 2-mercaptoethanol, transferred to PVDF membranes and probed with (4) secondary antibody alone, or (5) anti-light subunit mAb. Lanes 6–7: *E. dispar* trophozoites (10^5 per lane) electrophoresed by SDS-PAGE with 2-mercaptoethanol, transferred to PVDF membranes and probed with (6) secondary antibody alone, or (7) anti-light subunit mAb.

Because the anti-light subunit antibodies were produced against bacterially-expressed fusion proteins and a synthetic peptide, we tested whether these antibodies recognized the native lectin from *E. histolytica* (which is post-translationally modified by N-linked glycosylation and glycosylphosphatidylinositol anchor addition [5, 16]). The ability of mouse anti-light subunit antibodies to bind epitopes on the native lectin was analyzed by ELISA. Ninety-six well microtiter plates were coated with 0.1 μg of lectin per well and incubated with serial dilutions of anti-light subunit antiserum as described in Materials and methods. Both the anti-GST-LGL1 fusion protein antiserum and the antipeptide antisera bound to the affinity-purified lectin at dilutions up to 1:10 000 (Fig. 4). These results demonstrated that antibodies made from immunizations with the recombinantly expressed light subunit fusion protein and peptide recognize native lectin.

Despite the recognition of native lectin by the anti-light subunit antibodies, these antibodies did not dramatically alter amebic adherence to Chinese hamster ovary (CHO) cells. As can be seen in Table 1 saturating concentrations of the polyclonal and mAb had only modest effects on amebic adherence which were not statistically different from preimmune sera. Previously we had demonstrated that antibodies against the lectin heavy subunit inhibit or enhance Gal/GalNAc binding activity, depending on the epitope recognized [12, 16]. The lack of inhibition of

Table 1. Effect of anti-lectin light subunit monoclonal antibodies on amebic adherence to CHO cells.

Antibody	*Adherence (% control)*
Preimmune sera (1:100)	89 ± 7.9 ($n = 3$)
Anti-GST-LGL1 (1:100)	96 ± 18 ($n = 3$)
Anti-KLH-peptide (1:100)	79 ± 2.1 ($n = 3$)
Cocktail light subunit mAb	87 ± 13 ($n = 3$)

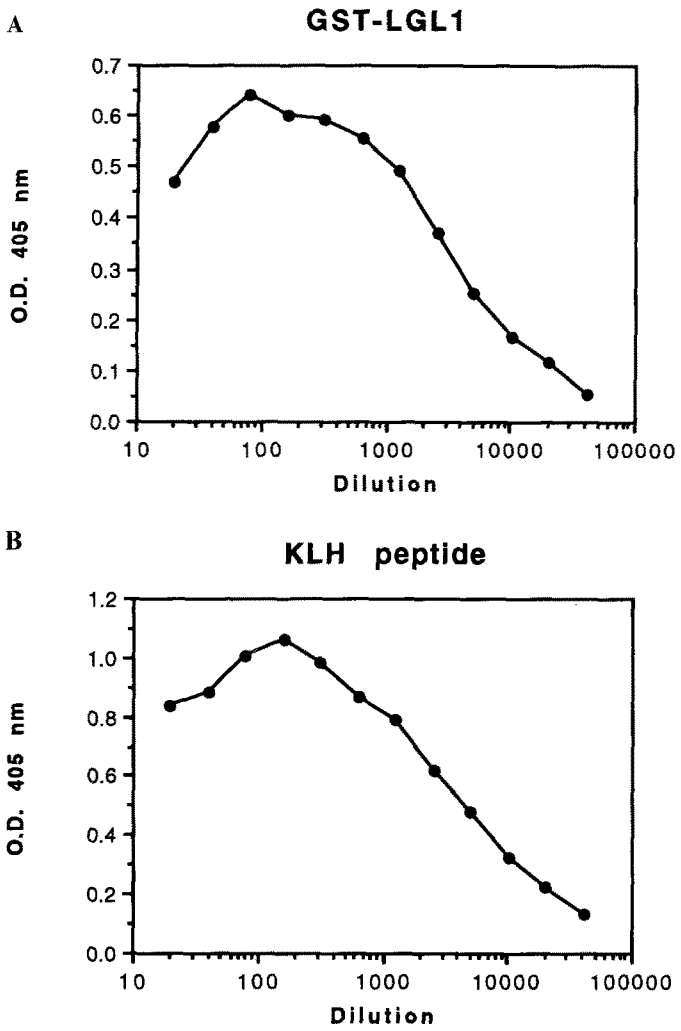

Figure 4. ELISA of purified lectin with mouse anti-light subunit antiserum. Serial dilutions of (A) A/J mouse anti-GST-LGL1 or (B) anti-light subunit peptide-KLH conjugate antiserum were incubated in 96-well microtiter plates coated with 0.1 μg per well of affinity-purified lectin. After washing, bound antibodies were detected with an anti-mouse IgG alkaline phosphatase conjugates (absorbance at 405 nm).

adherence with anti-light subunit antibodies is consistent with the carbohydrate recognition domain of the lectin residing on the heavy subunit.

Antibodies against the lectin light subunit have proven useful to demonstrate its invariant association with the lectin heavy subunit, its structural complexity in both *E. histolytica* and *E. dispar*, and its apparent lack of involvement in adherence. The functional significance of its structural complexity remains to be resolved.

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References

1. Ravdin JI, Guerrant RL (1981) *J Clin Invest* **68**:1305–13.
2. Chadee K, Petri WA Jr, Innes DJ, Ravdin JI (1987) *J Clin Invest* **80**:1245–54.
3. Braga LL, Ninomiya H, McCoy JJ, Eacker S, Wiedmer T, Pham C, Wood S, Sims PJ, Petri WA Jr (1992) *J Clin Invest* **90**:1131–37.
4. Mann BJ, Chung CY, Dodson JM, Ashley LS, Braga LL, Snodgrass TL (1993) *Infect Immun.* **61**:1772–78.
5. McCoy JJ, Mann BJ, Vedvick TS, Pak Y, Heimark DM, Petri WA Jr (1993) *J Biol Chem* **268**:24223–31.
6. Purdy JE, Mann BJ, Shugart EC, Petri WA Jr (1993) *Molec Biochem Parasitol* **62**:53–60.
7. Tannich E, Ebert F, Horstmann RD (1992) *Mol Biochem Parasitol* **55**:225–28.
8. McCoy JJ, Mann BJ, Vedvick TS, Petri WA Jr (1993) *Mol Biochem Parasitol* **61**:325–28.
9. Diamond LS, Harlow DR, Cunnick CC (1978) *Trans R Soc Trop Med Hyg* **72**:431–32.
10. Laemmli UK (1970) *Nature (London)* **227**:680–85.
11. Chapman MD, Sutherland WM, Platts-Mills TAE (1984) *J Immunol* **133**:2488–95.
12. Petri WA Jr, Chapman MD, Snodgrass T, Mann BJ, Broman J, Ravdin JI (1989) *J Biol Chem* **264**:3007–12.
13. Petri WA Jr, Jackson TFHG, Gathiram V, Kress K, Saffer LD, Snodgrass TL, Chapman MD, Keren Z, Mirelman D (1990) *Infect Immun* **58**:1802–6.
14. Petri WA Jr, Clark GC, Diamond LS (1994) *J Infect Dis* **169**:483–84.
15. Mann BJ, Vedvick T, Torian B, Petri WA Jr (1991) *Proc Natl Acad Sci USA* **88**:3248–52.
16. Petri WA Jr, Snodgrass TL, Jackson TFHG, Gathiram V, Simjee AE, Chadee K, Chapman MD (1990) *J Immunol* **144**:4803–9.